

Comparative study of *in vitro* digestibility of major allergen, tropomyosin and other proteins between Grass prawn (*Penaeus monodon*) and Pacific white shrimp (*Litopenaeus vannamei*)

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Abstract

BACKGROUND: Stability in simulated gastric fluid is supposed to be an important parameter for the estimation of food allergenicity. In the present study, the digestive stability of allergenic protein tropomyosin (TM) and other food proteins from Grass prawn and Pacific white shrimp in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) digestion assay system was investigated and comparatively studied by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), western blotting, and inhibition enzyme-linked immunosorbent assay (ELISA).

RESULTS: In the SGF system, proteins such as actin and myosin heavy chain (MHC) were rapidly degraded within a short period of time, while TM was relatively resistant to pepsin digestion. In the SIF system, MHC was also easily decomposed, while TM and actin were resistant to digestion. Western blotting using a specific polyclonal antibody against TM indicated that the degradation pattern of shrimp TM by SGF and SIF was almost unaffected by the presence of other myofibrillar proteins. Further study by IgE immunoblotting and inhibition ELISA using sera from crustacean-allergic patients indicated that IgE binding of TM was decreased.

CONCLUSION: Proteinase digestion is effective in reducing IgE binding of shrimp TM. It is also of interest to notice that Pacific white shrimp TM had higher digestion stability than Grass prawn TM. However, Pacific white shrimp TM revealed enhanced IgE binding over that of TM from Grass prawn and thus it is possibly more allergenic.

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Keywords: Grass prawn (*Penaeus monodon*); Pacific white shrimp (*Litopenaeus vannamei*); allergen; tropomyosin; digestibility; simulated gastric fluid; simulated intestinal fluid

INTRODUCTION

As the largest producer and consumer of aquatic products in the world, the production of shrimps in China reached 2.9 million tons in 2008.¹ Grass prawn (*Penaeus monodon*), also known as black tiger, is an important aquatic shellfish in south-east Asia and also an important export commodity in China.² On the other hand, Pacific white shrimp (*Litopenaeus vannamei*), also known as Whiteleg shrimp, is one of the most widely cultured shrimp in the world.³ There is a high consumer demand for Grass prawn and Pacific white shrimp, particularly in south-east Asia because of their delicious taste and nutritional value. However, as one of the eight major sources of food allergens proposed by the Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO), with the increasing production and consumption of shrimp and shrimp products, reports about allergic reactions induced by ingestion of such kind of foods are frequent.^{4,5}

Research has demonstrated that the major allergen of shrimp and crab is tropomyosin (TM). TM is a myofibrillar protein composed of two identical subunits with molecular masses of 35–38 kDa, each in α -helix form coiled around one another in the coiled-coil formation.^{5,6} Crustacean allergy is an IgE-mediated, type I allergy and a number of IgE-binding epitopes have been identified in the major allergen TM, though they may vary from one allergic individual to another.^{7,8} Furthermore, TM is extremely stable retaining its original allergenic activity even after heat treatment.^{6,9} More recently, we have cloned the full-length amino acid sequences of TM from three species of crab (Chinese mitten

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crab, mud crab and swimming crab) and found that the amino acid sequence identity of TMs among these crabs reached as high as 99.2%. We have also established an ELISA method to check and analyse the main allergen of shrimp TM.^{10,11} It is not surprising that crustacean TMs show high IgE cross-reactivity with one another.^{8,12}

It is generally regarded that for a native or denatured food protein to retain its allergenicity, the specific structure or linear amino acid sequence of the protein to which human IgE antibody is directed (i.e. the structural and linear epitopes) must survive food-processing treatments and *in vivo* digestion. The latter property suggested that for an allergen sensitising an individual via the gastrointestinal (GI) tract, it must endure degradation in the GI tract (such as resistance to low pH, bile salts and proteolysis), thus allowing allergen protein to reach the intestinal immune system.¹³ Accordingly, food allergens are generally resistant to pepsin digestion. The pepsin digestibility test has been regarded as one of the appropriate methods for evaluating the allergenicity of newly introduced proteins.⁴

Known food allergens are more stable under simulated gastric fluid (SGF) conditions than non-allergens.¹⁴ The investigation of stability of proteins within the gastrointestinal tract may provide prospective testing for allergenicity and could be a significant and valid parameter that distinguishes food allergens from non-allergens.¹³ Nevertheless, conflicting results have been obtained showing that food allergens are not necessarily more resistant to digestion than non-allergenic proteins.¹⁵ Bublin *et al.*¹⁶ found kiwi allergens Act d 1 precipitated in simulated gastric fluid at pH 2 and digestion of the aggregated protein proceeded slowly. Forty % of Act d 2 remained intact during gastric digestion. The digested allergen samples displayed nearly unchanged IgE-binding abilities.

At present, *in vitro* simulated digestion of allergen mainly focus on plant foods such as soybeans, peanuts, nuts, fruits, and vegetables, while studies on aquatic products are relatively few.¹⁷ Thus, in the present study, we focused on the digestive stability of the major shrimp allergen TM and other unproven allergenic proteins of shrimp muscle under SGF and simulated intestinal fluid (SIF) conditions. Furthermore, immunoassays were performed to make clear the effects of proteinase digestion on the IgE binding of shrimp TM.

MATERIALS AND METHODS

Shrimp and chemicals

Grass prawn (*Penaeus monodon*) and Pacific white shrimp (*Litopenaeus vannamei*) was purchased alive at Jimei market, Xiamen. After washing, shrimp muscle was obtained and used immediately.

Protein standards for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were purchased from Fermentas (Vilnius, Lithuania). Prestained protein standards for western blotting were from New England BioLabs (Beverly, MA, USA). Rabbit anti-mud crab TM polyclonal antibody was prepared in our laboratory.¹⁰ Horseradish peroxidase (HRP)-labelled rabbit anti-rat IgG was from DAKO (Glostrup, Denmark). HRP-labelled goat anti-human IgE was from Kirkegaard & Perry Laboratories (Gaithersburg, MD, USA). HRP-labelled goat anti-rabbit IgG and 3,3'-diaminobenzidine tetrahydrochloride (DAB) were from Pierce (Rockford, IL, USA). Serine proteinase inhibitor pefabloc SC was from Merck (Darmstadt, Germany). *o*-Phenyldiamine, porcine trypsin and bovine α -chymotrypsin were purchased from Sigma (St Louis, MO, USA). Porcine pepsin was prepared in our laboratory as previously described.¹⁸ The proteolytic

activity of pepsin was estimated by measuring trichloroacetic acid precipitable bovine haemoglobin after hydrolysis for 30 min. One unit of pepsin activity was defined as an increase of absorbance of 1.0 at 280 nm for 30 min incubation at 37 °C. The activity of pepsin for hydrolysing hemoglobin was 318.2 U and the specific activity of pepsin was 272 U mg⁻¹. Other reagents were all of analytical grade.

Human sera

Sera from five patients were obtained from the Hospital of Jimei University. The patients with ages of 18 to 22 years were all proposed to have crustacean allergy on the basis of their clinical history of immediate hypersensitivity reactions, such as urticaria and diarrhoea, after ingestion of crustaceans. Written informed consent was obtained from each patient. These patient sera were used as a pooled positive sample or individually, and sera from non-allergic individual used as negative control. All sera were stored at –80 °C until used.

Preparation of myofibrillar proteins

Grass prawn and Pacific white shrimp myofibrillar proteins were prepared individually as described.¹⁹ Briefly, shrimp muscle was minced and homogenised with five volumes of ice-cold 20 mmol L⁻¹ Tris-HCl buffer (pH 7.5) using a homogeniser (Kinematica, Lucerne, Switzerland). The homogenisation process was performed using a polytron of PT-2120 at speed indicator 15, and the operation was carried out three times for 30 s each with an interval of 1 min. The homogenate was centrifuged at 8000 $\times g$, for 10 min at 4 °C. The precipitate was collected and resuspended in five volumes of 20 mmol L⁻¹ Tris-HCl buffer. After four repeated cycles of homogenisation and centrifugation, the resulting precipitate was dissolved in 100 mmol L⁻¹ Tris-HCl buffer (pH 7.5) containing 0.5 mol L⁻¹ NaCl and was regarded as myofibril extract.

Purification of shrimp TM and protein content determination

The purification of shrimp TM was carried out as described.^{10,11} Briefly, an acetone powder of myofibrillar proteins was prepared from shrimp muscle as reported by Huang and Ochiai²⁰ and extracted with 10 volumes of 20 mmol L⁻¹ Tris-HCl buffer (pH 7.5) containing 1 mol L⁻¹ KCl and 10 mmol L⁻¹ 2-mercaptoethanol. The homogenate was centrifuged at 2000 $\times g$ for 20 min, the supernatant was isoelectric precipitated at pH 4.5 with HCl and the precipitate was dissolved in 20 mmol L⁻¹ Tris-HCl (pH 7.5) and successively fractionated with 40–60% saturation of (NH₄)₂SO₄. After centrifugation at 20 000 $\times g$ for 20 min, the precipitate was collected and redissolved in 20 mmol L⁻¹ Tris-HCl (pH 7.5) followed by filtering off the residue. Finally, the small amount of impurity was removed by heating in a boiling water bath for 10 min followed by immediate cooling in ice water and centrifugation at 20 000 $\times g$ for 20 min. The supernatant containing TM with high purity was used for experiments or stored at –80 °C until used.

The protein concentration of myofibrils and purified shrimp allergen TM was determined by measuring the absorbance at 280 nm or with the Lowry method²¹ using bovine serum albumin as the standard.

Simulated gastric fluid digestion stability assay

The digestibility of purified TM and myofibrillar proteins in SGF was prepared as described in the *United States Pharmacopoeia*²²

and consisted of porcine pepsin (272 U mg^{-1}) at pH 1.2. The total volume of reaction solution was 1 mL. For each sample, a centrifuge tube (1.5 mL) containing SGF was preheated at 37°C for 15 min before the adding of the tested protein. A ratio of 0.33 U of pepsin activity μg^{-1} protein was selected for all tests (1:50 pepsin to protein, w/w). Digestion was performed at 37°C with continuous rocking. An aliquot (100 μL) of the digest was withdrawn at different time intervals (0.5, 1, 2, 5, 10, 15, 30 and 60 min) and was immediately terminated by adding of 30 μL of 200 mmol L^{-1} Na_2CO_3 and 26 μL of 4 $\times$ Laemmli buffer. The samples were heated at 95°C for 10 min and analysed by SDS-PAGE or western blotting. For the 0 min sample preparation, protein samples were mixed with SGF that was neutralised with Na_2CO_3 . After adding Laemmli buffer, samples were then heated for SDS-PAGE analysis. In the control experiments, each protein sample was dissolved in the reaction buffer without pepsin and then treated as described above. All the experiments were repeated in triplicate or more times and no obvious differences were identified.

Simulated intestinal fluid digestion stability assay

For intestinal digestion, porcine trypsin and bovine α -chymotrypsin were used. SIF was prepared as described in the *United States Pharmacopoeia*²² and consists of trypsin or α -chymotrypsin in 0.05 mol L^{-1} KH_2PO_4 , pH 7.5. The final volume of reaction solution was 1 mL. For each sample, a centrifuge tube containing 1 mL of simulated intestinal fluid (SIF) was preheated at 37°C prior to the addition of test protein. The enzyme activity of trypsin and α -chymotrypsin was 325 U mg^{-1} protein and 62 U mg^{-1} protein, respectively.

Digestion conditions were as follows: a ratio of 0.20 U of trypsin activity μg^{-1} protein was selected for all tests (1:50 trypsin to protein, w/w), and a ratio of 0.04 U of chymotrypsin activity μg^{-1} protein was selected for all tests (1:50 chymotrypsin to protein, w/w). Digestions were performed at 37°C with continuous rocking. An aliquot (100 μL) of the digest was withdrawn at different time intervals (0, 1, 15, 30, 60, 120, 180 and 240 min), and 26 μL of the sample buffer was immediately added. The reaction was stopped by adding serine proteinase inhibitor pefabloc SC to a final concentration of 5 mmol L^{-1} and heating at 95°C for 10 min followed by SDS-PAGE analysis. For 0 min sample preparation, each sample was mixed with SIF that had been inactivated by pefabloc SC and boiling for 5 min. In the control experiments, each protein sample was dissolved in the reaction buffer that did not contain corresponding enzyme and then treated as described above. All the experiments were repeated in triplicate.

SDS-PAGE analysis and western blotting

SDS-PAGE was performed under reducing conditions according to the method of Laemmli.²³ Samples were separated in 12% polyacrylamide gels with 5% stacking gel and the gels were stained for protein with Coomassie brilliant blue R-250 (CBB).

Western blotting was carried out as described by Towbin *et al.*²⁴ Briefly, protein samples were separated on the gels and electrotransferred onto nitrocellulose membranes. Nonspecific protein sites were blocked with 5% non-fat milk in Tris-HCl buffered saline (TBS is 20 mmol L^{-1} Tris-HCl, pH 7.5, containing 0.145 mol L^{-1} NaCl). The membrane was incubated with rabbit anti-mud crab TM polyclonal antibody (1:2000 dilution) at room temperature for 2 h and washed with TBST (TBS, 0.05% Tween-20). After incubation for 1 h with HRP-labelled rabbit anti-rat IgG or goat anti-rabbit IgG secondary antibodies (1:2000 dilution), the

membranes were washed extensively with TBST and detected with DAB.

For the detection of IgE-binding activity of TM and its digestive products, the membrane was incubated with human serum (1:7 dilution) at 4°C for 16 h. HRP-conjugated goat anti-human IgE antibody (1:2000 dilution) was then allowed to react with the membrane at room temperature for 1 h followed by washing and detection by DAB.

Inhibition ELISA

Polystyrene 96-well ELISA plates (Nunc Maxisorb; Nunc, Roskilde, Denmark) were coated with TM (200 ng well^{-1}) at 4°C for 16 h using 15 mmol L^{-1} sodium carbonate (pH 9.6) as the coating buffer. Coated plates were washed five times with TBST and then blocked with 200 μL blocking buffer (0.5% bovine serum albumin in TBST) at 37°C for 2 h. In another microtitre plate, 100 μL human sera (diluted 1/20 with 0.1% bovine serum albumin in TBS) was mixed with equal volumes of digested TM (respectively with pepsin, trypsin and chymotrypsin) as inhibitor in the concentration range of 0.02–40 $\mu\text{g mL}^{-1}$. After incubation at 37°C for 1 h, each 50 μL of the sera-TM mixture was transferred to the ELISA plate coated with TM and further incubated at 37°C for 1 h. Plates were washed with TBST, followed by reaction with HRP-conjugated goat anti-human IgE antibody (1:2000 dilution) for 2 h at 37°C . The colour reaction was developed using 0.05 mol L^{-1} phosphate-citrate buffer (pH 5.0) containing 0.1% *o*-phenyldiamine and 0.03% H_2O_2 and terminated by addition of 2 mol L^{-1} sulfuric acid. The developed colour was measured by a microplate reader (Benchmark 96; Bio-Rad Laboratories, Hercules, CA, USA) at 490 nm. The loss of specific IgE-binding ability of patients' sera resulting from the treatment with human sera and digested TM as inhibitor was represented by calculating the inhibition rate using the formula:²⁵ inhibition rate (%) = $(X - Y)/(X - Z) \times 100$, where X was the absorbance of patients' sera without inhibitors, and Y and Z were the absorbance of patients' and control sera treated with various concentrations of inhibitors, respectively. All determinations were duplicated and variations between the two assays were always <10%. The mean values were used.

RESULTS

Digestibility of purified TM by SGF and SIF

The digestibility of the purified Grass prawn and Pacific white shrimp allergen TM was examined by SDS-PAGE. Purified TM revealed a band corresponding to the molecular mass of about 36 kDa (Figs 1 and 2). Digestive patterns of TM by pepsin, trypsin and chymotrypsin were quite different. Based on the time that foods remain in the human digestive system, the digestion time in stomach and intestine was designated as 1 h and 4 h, respectively. The digestive result of Grass prawn TM is shown in Fig. 1. Grass prawn TM was relatively stable in SGF even after incubation for 1 h (Fig. 1A), but could be digested completely after 4 h by trypsin (Fig. 1B) and chymotrypsin (Fig. 1C). In the SIF digestion assay of Grass prawn TM, whether by trypsin or chymotrypsin, many minor degraded fragments appeared (Fig. 1B and C). Two major proteolytic fragments (28 and 22 kDa) could clearly be detected.

The digestive results of the Pacific white shrimp are shown in Fig. 2. Compared to that of Grass prawn TM, Pacific white shrimp TM was relatively stable. Only minor fragments appeared after 60 min in SGF (Fig. 2A). With the increasing of digestion time, only

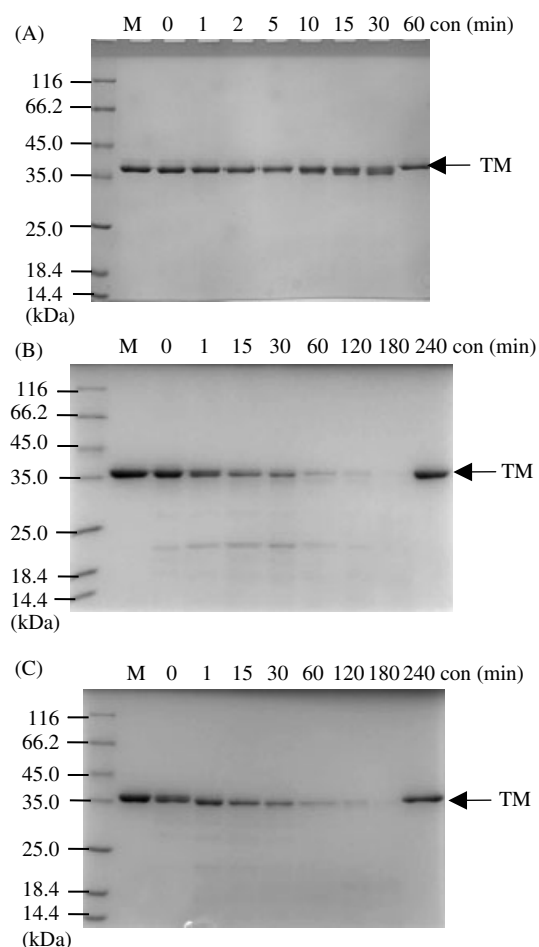


Figure 1. SDS-PAGE analysis of SGF and SIF digestion on Grass prawn TM. Proteinases used were pepsin (A), trypsin (B) and α -chymotrypsin (C). Enzyme digestion was performed as described in Materials and Methods followed by SDS-PAGE. After electrophoresis, proteins were visualised by staining with CBB. In the control experiments (con) proteinase was excluded. Molecular masses of the protein markers (M) are shown at the left edges.

a small amount of TM was further degraded by trypsin (Fig. 2B) and chymotrypsin (Fig. 2C), and the original TM band still remained even after 4 h. The fragment with molecular mass 22 kDa was the major product of the degraded product of Pacific white shrimp TM. As digestion time increased, the digestion-resistant 22 kDa fragment progressively intensified (Fig. 2B and C).

Digestibility of shrimp myofibrillar proteins by SGF and SIF

Myofibrils mainly consist of myosin heavy chain (MHC, 200 kDa), actin (42 kDa) and other minor proteins such as α -actinin (100 kDa), TM, troponins and myosin light chain. As TM represents only a small portion of shrimp muscular proteins, it is necessary to compare the digestive stability of TM with other non-allergenic proteins or proteins with unproven allergenicity in myofibrils.

The results of the Grass prawn myofibrillar proteins *in vitro* digestion is shown in Fig. 3. MHC and actin could be effectively degraded after 1 min under the action of digestive enzymes, and could be decomposed completely with the increasing time. In the SGF reaction system, actin could not be detected even in as short as 1 min. In contrast, intact TM was not completely digested under the same SGF conditions for 60 min, while a major fragment

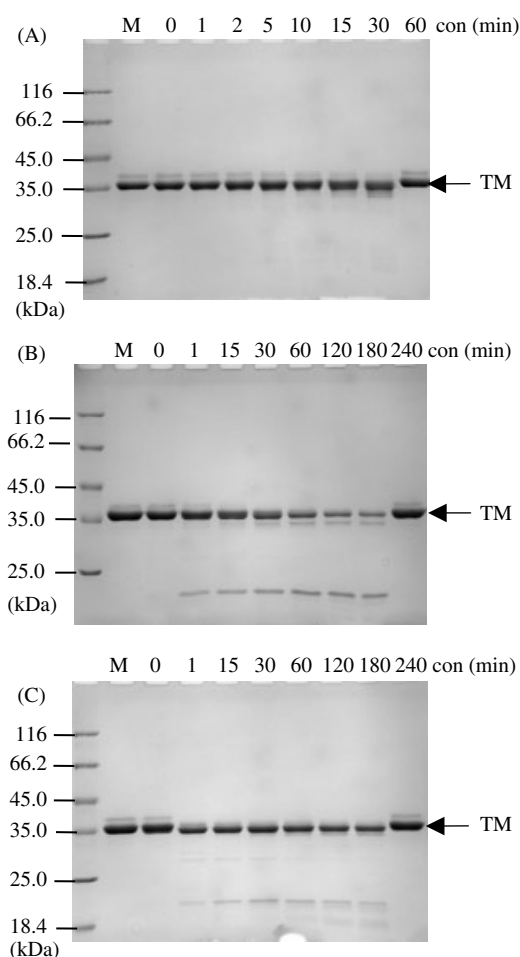


Figure 2. SDS-PAGE analysis of SGF and SIF digestion on Pacific white shrimp TM. Proteinases used were pepsin (A), trypsin (B) and α -chymotrypsin (C). Enzyme digestion was performed as described in Materials and Methods followed by SDS-PAGE as in Fig. 1. Molecular masses of the protein markers (M) are shown at the left edges.

of approximately 34 kDa which was produced after 5 min and survived digestion up to 60 min was observed (Fig. 3A). Actin was also degraded easily in SIF. The band of actin weakened gradually by trypsin treatment, and some steady fragments of 37 kDa were produced after 15 min while TM was hardly degraded (Fig. 3B). Actin was broken down completely after 2 h by chymotrypsin digestion (Fig. 3C). As shown in Fig. 3, degraded protein fragments gradually increased with the prolongation of digestion time. It should be noticed that most of these fragments were in the molecular masses range between 200 kDa and 66 kDa which are quite possibly degraded products of MHC, although the possibility that some of them were from much larger sized proteins such as titin (3000 kDa) and nebulin (600–800 kDa) should not be excluded. On the other hand, a new protein band (34 kDa) which is quite possibly the proteolytic fragment of TM was observed (Fig. 3A). This result agreed well with that using purified TM as shown in Fig. 1A, suggesting the presence of other proteins did not interfere with the digestion of TM by SGF.

The degradation pattern of the Pacific white shrimp myofibrillar proteins in SGF was almost the same with that of Grass prawn myofibrillar proteins (Fig. 4A). In SIF digestion, the difference was that the Pacific white shrimp actin was broken down completely by trypsin in 2 h, and the degradation band of 37 kDa gradually

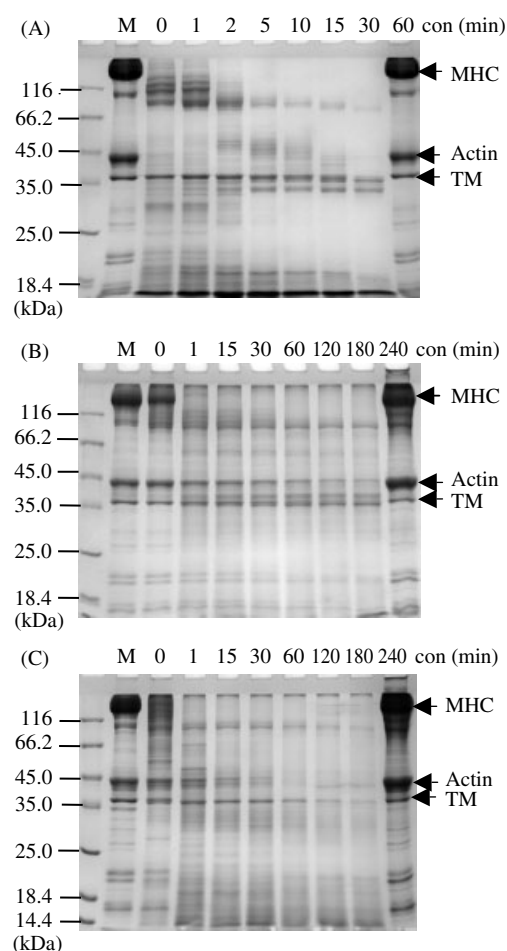


Figure 3. SDS-PAGE analysis of SGF and SIF digestion on Grass prawn myofibrillar proteins. Proteinases used were pepsin (A), trypsin (B) and chymotrypsin (C). Enzyme digestion was performed as described in Materials and Methods followed by SDS-PAGE as in Fig. 1. Molecular masses of the protein markers (M) are shown at the left edges.

increased from 1–120 min, while the band of 37 kDa gradually weakened thereafter. Pacific white shrimp TM was also gradually broken down by trypsin, and the original protein band was very weak after 3 h treatment (Fig. 4B). The digestion pattern of Pacific white shrimp myofibrillar proteins by chymotrypsin was almost the same as that of Grass prawn myofibrillar proteins (Fig. 4C). Both the original bands of TM were detectable even after 4 h digestion.

Western blotting analysis of individual proteins

Myofibrillar proteins with larger molecular mass such as MHC, nebulin and titin present sources of fragment products that are difficult to ascertain by SDS-PAGE. To clarify the identity of the degraded myofibrillar protein fragments, the degradation of allergen TM in the myofibril extract by SGF and SIF was specifically investigated by western blotting.

In contrast to other proteins, Grass prawn TM was relatively resistant to SGF digestion, and only partial degradation was observed after 60 min (Fig. 5A). Grass prawn TM also revealed high resistance to the digestion by trypsin and chymotrypsin, and only minor amount of fragments with sizes of 34 kDa and 22 kDa appeared after trypsin digestion for 2 h (Fig. 5B). In the presence of chymotrypsin, TM was also digested and produced

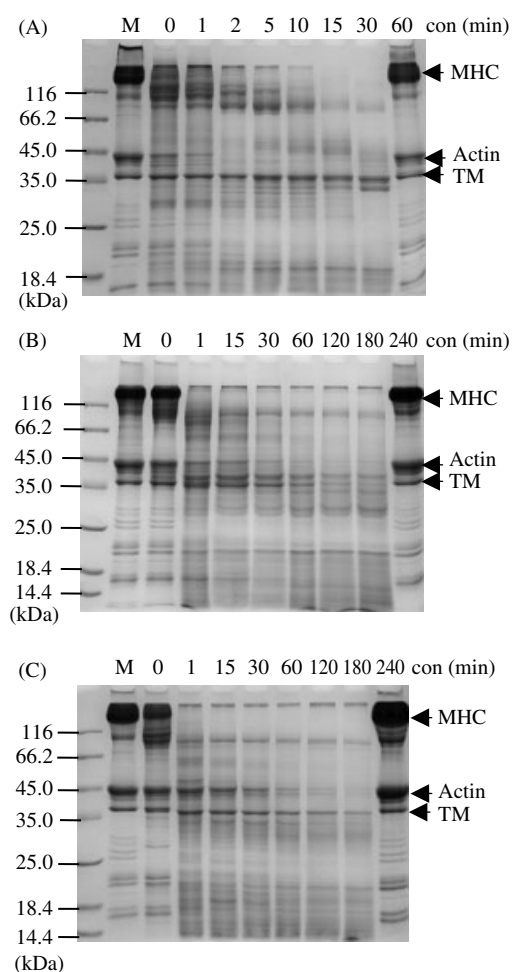


Figure 4. SDS-PAGE analysis of SGF and SIF digestion on Pacific white shrimp myofibrillar proteins. Proteinases used were pepsin (A), trypsin (B) and chymotrypsin (C). Enzyme digestion was performed as described in Materials and Methods followed by SDS-PAGE as in Fig. 1. Molecular masses of the protein markers (M) are shown at the left edges.

random fragments in the range of 32–25 kDa, but the original TM band still existed even after 4 h (Fig. 5C). It is noteworthy that the digestive results of purified TM by means of SDS-PAGE (Fig. 1) were in accordance with that by western blotting (Fig. 5), suggesting the degradation pattern of TM by SGF and SIF was not affected by the presence of other myofibrillar proteins.

As shown as Fig. 6A, the degradation pattern of Pacific white shrimp TM in myofibrillar proteins by SGF was similar to that of Grass prawn TM. The degradation fragments of 35, 28 and 22 kDa were produced, and the TM was not broken down completely even after 4 h digestion by SIF (Fig. 6B and C). In short, the result as detected by western blotting (Fig. 6) was in agreement with that of SDS-PAGE (Fig. 2) using purified Pacific white shrimp TM.

IgE binding of TM by proteinase digestion

The IgE binding of TM by proteinases (pepsin, trypsin and chymotrypsin) digestion was further analysed by means of immunoblotting (Fig. 7). Western blotting with a serum pool of crustacean hypersensitive patients showed that their IgE antibodies positively reacted with the original TM band (36 kDa) and protein bands with size of 34 kDa. Smaller fragments (28 and 22 kDa) could be detected in Pacific white shrimp (Fig. 7B)

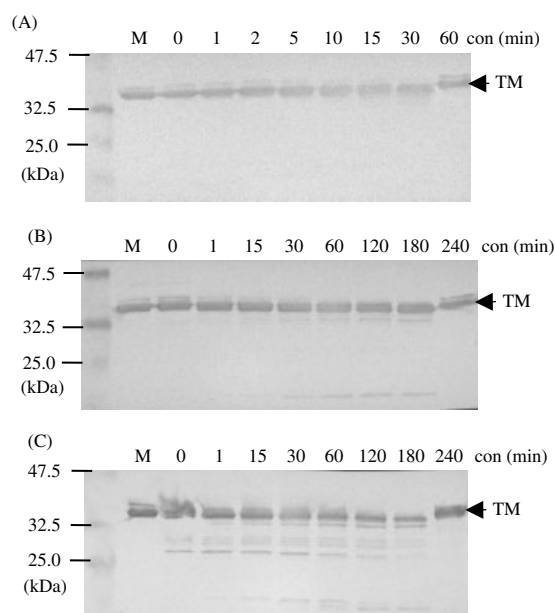


Figure 5. Western blotting detection of the degradation of TM from Grass prawn in SGF and SIF. Proteinases used were pepsin (A), trypsin (B) and chymotrypsin (C). Samples were separated by SDS-PAGE followed by immunological detection using a polyclonal antiserum against mud crab TM. Molecular masses of the protein markers (M) are shown at the left edges.

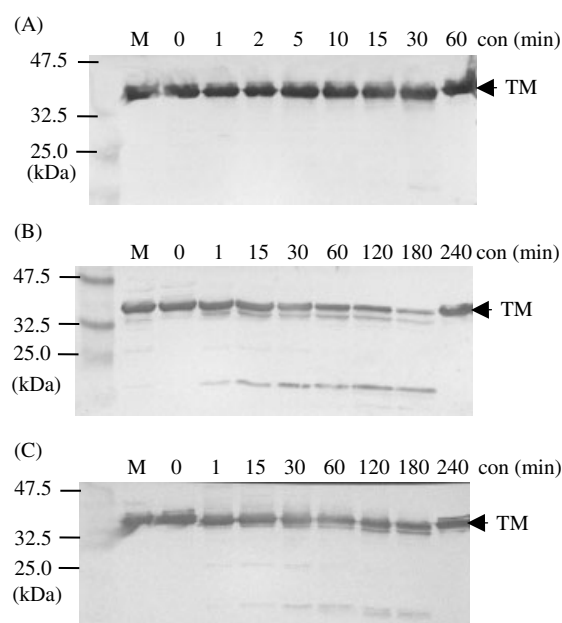


Figure 6. Western blotting detection of the degradation of TM from Pacific white shrimp in SGF and SIF. Proteinases used were pepsin (A), trypsin (B) and chymotrypsin (C). Samples were separated by SDS-PAGE followed by immunological detection using a polyclonal antiserum against mud crab TM. Molecular masses of the protein markers (M) are shown at the left edges.

but not in Grass prawn (Fig. 7A). Possibly, IgE-binding epitopes in these fragments from Grass prawn were destroyed by trypsin and chymotrypsin digestion.

The IgE binding of digestive products of Grass prawn and Pacific white shrimp was assessed using inhibition ELISA (Fig. 8). Briefly, Grass prawn or Pacific white shrimp extracts were coated on 96-well microtitre plates. The digestive products of either Grass prawn or Pacific white shrimp were pre-incubated with serum from crustacean allergic patients. Compared with undigested sample, the IgE binding of all digested samples was obviously reduced by proteinases treatment. In Fig. 8, the protein concentration ($2 \mu\text{g mL}^{-1}$) of the products of Grass prawn TM digested by pepsin needed to exert 50% inhibition was approximately 10-fold higher than that ($0.2 \mu\text{g mL}^{-1}$) of Pacific white shrimp. While the inhibition rate of 50% for digestive products ($0.6 \mu\text{g mL}^{-1}$) of Grass prawn by chymotrypsin was approximately 30-fold higher than that ($0.02 \mu\text{g mL}^{-1}$) of Pacific white shrimp. But the inhibition rate of 50% for digestive products ($70 \mu\text{g mL}^{-1}$) of Grass prawn by trypsin was similar to that ($40 \mu\text{g mL}^{-1}$) of Pacific white shrimp. Proteinases treatment may cause lower IgE binding because some of the IgE-binding epitopes may be destructed or modified.

DISCUSSION

The physicochemical properties of most food allergens confer stability to proteolytic digestion, which increases the probability of reaching the intestinal mucosa, where absorption and interaction with the immune system can occur.¹⁶ The different TM digestion patterns *in vitro* by pepsin, trypsin and chymotrypsin are the result of different cleavage specificities for peptide bonds of these three different proteinases. Pepsin prefers to cleave peptide bonds following Phe or Tyr residues. Trypsin reveals a well-defined specificity, cleaving next to the hydrophilic amino acid residues Lys

and Arg at the P_1 position. Chymotrypsin has a broader specificity, preferring cleavage next to aromatic amino acids (Tyr, Trp, Phe) and Leu in the P_1 position, and to a much lower extent next to a few other amino acids.²⁶

Results from *in vitro* simulated digestion studies with purified protein are routinely used to assess the sensitisation of potential allergens. However, the persistence of purified protein in simulated digestion may be a poor predictor of the allergenic status of a protein, potentially due to the presence of other food proteins, which can be significant *in vivo*.¹⁷ In the present study, the digestibility of TM in the presence of other myofibrillar proteins was evaluated by immunoassay with rabbit anti-TM polyclonal antibody and sera from patients allergic to crustaceans. Although the antibody against TM was prepared using mud crab tropomyosin, this protein is well conserved in evolution, and actually the polyclonal antibody was effective in detecting shrimp TM as well as certain of its proteolytic fragments. Both SDS-PAGE using purified shrimp TM and western blotting using myofibrillar proteins indicated that TM was relatively higher resistant to digestion than other myofibrillar proteins, and Pacific white shrimp TM revealed higher digestion stability than Grass prawn TM. These results suggest that the ability of TM to survive gastrointestinal digestion structurally intact increases its potential to sensitise.

Our present study also indicates that the enzyme-to-substrate ratio was influential in the stability of shrimp TM. When the pepsin to TM ratio (weight to weight) increased from 0.02 to 1, the protein was highly unstable. When the pepsin/test protein ratio increased to 5, neither the original protein nor degradation fragments were detectable after 10 min (data not shown). It is widely acknowledged that human digestion is variable from person to person,^{13,27,28} so it is not easy to choose the optimal pepsin-to-substrate ratio which accurately reflects the physiological situation. For protein digestion analysis, it is more reasonable that

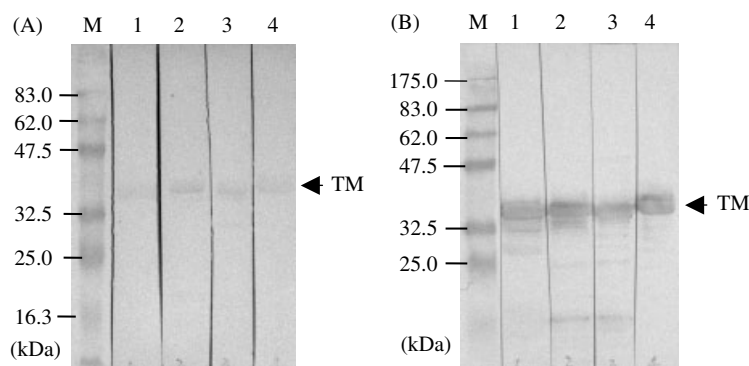


Figure 7. Western blotting using human serum to detect the IgE binding of TM from Grass prawn (A) and Pacific white shrimp (B) after digestion by SGF and SIF. 1, purified TM; 2, digested with pepsin; 3, digested with trypsin; 4, digested with chymotrypsin. Digestion time was 60 min. Molecular masses of the protein markers (M) are shown at the left edges.

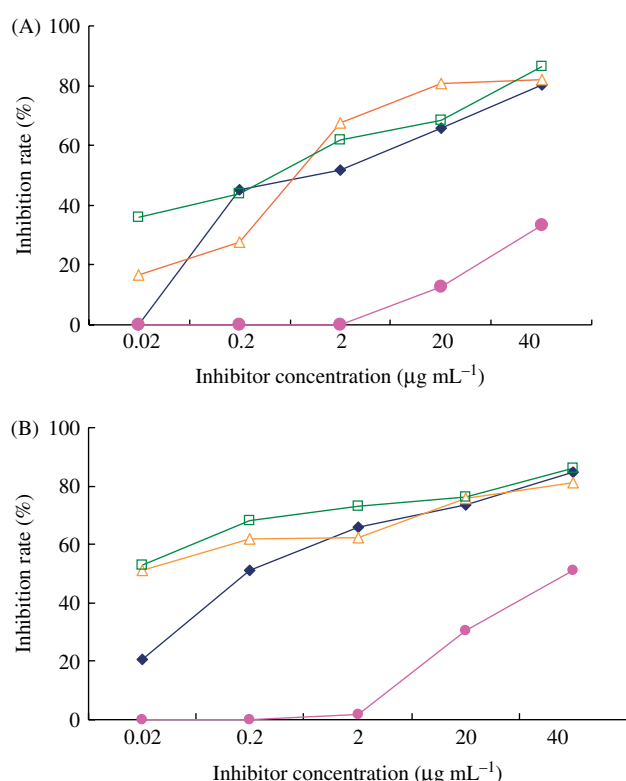


Figure 8. Inhibition ELISA of TM digestive samples from Grass prawn (A) and Pacific white shrimp (B). The digestive samples by pepsin (Δ), trypsin ($\bullet$), chymotrypsin ($\blacklozenge$) for 1 h, 4 h, 4 h, respectively and undigested TM ($\square$) were diluted 1:5, 1:10, 1:100, 1:1000, 1:10 000 and used as inhibitor solutions. Pooled patient serum (1:20 dilution) was incubated with an equal volume of inhibitor solution at 37 °C for 1 h followed by ELISA. The initial protein concentration of each sample was 200 $\mu\text{g mL}^{-1}$.

the amount of proteinases used should be based on proteolytic activity but not on weight. In the present study, porcine pepsin was prepared and its specific activity was 272 U mg^{-1} according to our definition and it reached 8185 U mg^{-1} according to the method of Sigma Chemical Co. Because the porcine pepsin we prepared was of higher activity than commercial one (800–4500 U mg^{-1}), a lesser amount of pepsin was used for experiment and this may be the main reason that the pepsin band could not be observed on SDS-PAGE.

Proteolytic digestion substantially decreases the potential of food proteins to bind IgE, which increases the threshold dose of allergens required to elicit symptoms in patients with food allergy.^{29,30} Compared with undigested sample, the IgE binding of all digested samples was obviously reduced by proteinase treatment, suggesting proteinase treatment such as by trypsin and chymotrypsin is an appropriate way to reduce TM IgE binding. TM from Pacific white shrimp showed enhanced IgE binding over that of TM from Grass prawn, suggesting Pacific white shrimp may be more antigenic.

It should be remembered that our present study only measured the IgE binding which may not completely equate to allergenicity. This is because the determination of allergenicity requires divalent reactivity of the allergenic protein to cross-link IgE antibodies on the surface of mast cell or basophile membranes. For partially digested proteins, some fragments may just contain one IgE binding site which may be able to bind IgE but not able to lead mediators release from mast cells or basophiles. Thus, to further evaluate the alterations of TM after proteinase treatment, experiments such as the release of histamine from basophiles and skin prick test are required. In addition, determining the amount of undigested TMs that survive gastrointestinal digestion and induces food allergy is needed.

CONCLUSIONS

The digestibility of Grass prawn and Pacific white shrimp major allergen TM by SGF and SIF was studied. TM was resistant to pepsin while relatively susceptible to trypsin and chymotrypsin digestion. Both SDS-PAGE using purified TM and western blotting using myofibrillar proteins indicated that the degradation pattern of TM by SGF and SIF was not affected by the presence of other myofibrillar proteins. Inhibition ELISA results revealed that proteinase treatment is an effective way for reducing the IgE binding of TM. By comparison, Pacific white shrimp TM revealed higher digestion stability than Grass prawn TM.

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